

Screening of Type II Restriction Endonucleases

I. Isolation and Characterization of Enzymes from Fifteen Bacterial Strains

Hiroyuki SUGISAKI*, Yoshihiko MAEKAWA*,
Susumu KANAZAWA* and Mituru TAKANAMI*

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One hundred and forty-seven bacterial strains were surveyed for the presence of type II restriction endonucleases, and eighteen species of enzymes were successfully isolated from fifteen strains in which enzyme activities were identified. Based on analysis of the restriction patterns generated from viral and plasmid DNAs and of the sequences around the cleavage-sites, four of enzymes named *AatII*, *BanII*, *FokI* and *MluI* were found to have new specificities. The remaining fourteen enzymes, named *AatI*, *AtuIAMI*, *BanI*, *BanIII*, *BprI*, *EcoICRI*, *GglI*, *GinI*, *MauI*, *PaiI*, *PanI*, *PflI*, *PpuI*, and *SpaI*, were isoschizomers of known enzymes.

KEY WORDS: Restriction endonuclease/ Recognition sequence/ Isoschizomer

I. INTRODUCTION

Type II restriction endonucleases are a class of enzymes which introduce double-stranded cleavages at unique sites of DNA by recognizing specific sequences. This type of enzymes have only been identified in bacterial strains and their specificities are known to be strain-specific. Since the collection of different enzymes is of course advantageous for gene cloning and DNA dissection, many bacterial strains have extensively been surveyed during the last several years, and more than seventy species of enzymes with different specificities have now been isolated.^{1,2)} The recognition sequences of these enzymes are limited to tetra- to hexa-nucleotides, and most of them have two-fold rotational symmetry. Assuming that enzymes corresponding to all possible sequences of tetra- to hexa-nucleotides with such palindromic structures are present in microorganisms, the number of enzymes are expected to be more than one hundred fifty. Based on this assumption, we carried out screening of new enzymes on bacterial strains which have not been examined yet. As a consequence, we identified enzyme activities in fifteen strains out of one hundred forty-seven strains examined. Enzymes were purified from respective strains and their cleavage-site specificities were analyzed.

II. MATERIALS AND METHODS

Bacterial strains and culture conditions

The sources and strain names used for screening are listed in *Table I*. *Acetobacter*

* 梶崎 弘幸, 前川 宜彦, 金沢 進, 高浪 満: Research Facility for Nucleic Acids, Institute for Chemical Research, Kyoto University, Uji, 611.

and *Gluconobacter* strains were obtained from Dr. T. Tochikura of Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, some of *Pseudomonas* and *Streptomyces* strains from Toyoboseki Co., and all other strains from Dr. K. Soda of this Institute, respectively. The compositions of culture media were as follows:

- a) *Acetobacter* and *Gluconobacter* strains; 0.5% glucose, 1.5% glycerol, 0.5% yeast extract, 0.5% bacto-tryptone, and 0.1% malt-extract (pH 7.2).
- b) *Lactobacillus* and *Streptococcus* strains; 0.85% yeast extract, 1.1% glucose, 0.2% KH_2PO_4 , 10% tomato juice, 0.1% Tween 80 and 2% CaCO_3 (pH 7.2).
- c) *Micrococcus*, *Mycobacterium*, *Pseudomonas* and *Serratia* strains; 1% meat extract, 1% bacto-tryptone, 2% glucose and 5% NaCl (pH 7.0).
- d) *Streptomyces* strains; 2% nutrient broth (Difco), 0.2% yeast extract, 0.8% NaCl, and 0.5% glucose (pH 7.2).
- e) All other strains; 1% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl and 0.1% glucose (pH 7.2).

Strains were grown at either 30°C (a to e) or 37°C (c and e) to a late logarithmic phase by vigorous shaking in a NBC-rotary shaker. The cells were then harvested by centrifugation, washed once with buffered saline and stored at -70°C .

Viral and plasmid DNAs and other enzymes

Phage T4dC and lambda DNAs and plasmids, pAO2, pAO43 and pBR322, were prepared as in the previous paper.^{3,4)} Phage ϕX174 RF-I and SV40 DNAs were purchased from Bethesda Research Laboratories. The sources of other enzymes used for restriction and sequence analyses have been described previously.⁵⁾

Purification of enzymes

Purification of enzymes was carried out by the procedures essentially identical to those previously used.³⁻⁵⁾ Ten grams of the frozen cells were suspended in 50 ml of 0.05 M tris-HCl (pH 7.5) and 2 mM 2-mercaptoethanol, disrupted by sonication in a Tomy Ultrasonic Disruptor and centrifuged for 60 min at 30,000 rpm in a Spinco 50Ti rotor. The high-speed supernatant was made 0.1 M NaCl, and then a 10% stock solution of polyethyleneimine, dissolved in water and neutralized, was added to 1%. The precipitate containing mostly nucleic acids was removed by centrifugation, and to the supernatant was added solid ammonium sulfate to 70% saturation. The resulting precipitate was collected by centrifugation, dissolved in 0.01 M potassium phosphate buffer (pH 7.5) and 1 mM 2-mercaptoethanol, and dialyzed against the same buffer. The solution was applied on a phospho-cellulose column (Whatman, 1 cm $\times$ 20 cm) and chromatographed with a linear gradient of KCl, and type II-enzyme activities were assayed. The active fractions were applied on a DE52-cellulose column (Whatman, 1 cm $\times$ 15 cm) and chromatographed with a linear gradient of KCl. Further purification of enzymes was carried out by heparin-agarose column chromatography.⁶⁾

Assay of enzyme activities

One to five μl of fractions were added to 30 μl reaction mixtures containing 0.01 M tris-HCl (pH 7.5), 7 mM MgCl_2 , and 1 μg of viral or plasmid DNAs. After

incubation for 30 min to 2 hrs at 37°C, reaction was terminated by adding 5 μ l of 0.05 M EDTA, 15% Ficoll and 0.02% bromophenol blue. The solutions were electrophoresed for 2 to 4 hrs at 10 V/cm on horizontal slab gels of 1% agarose, dissolved in 90 mM tris-borate buffer (pH 8.3), 2.5 mM EDTA and 0.5 μ g/ml ethidium bromide.

Nucleotide sequence determination

Sequence analysis was carried out by the method of Maxam and Gilbert.⁷⁾ The two dimensional mobility-shift method, which uses electrophoresis and homochromatography, was applied for identification of unique 5'-terminal sequences in a mixture of restriction fragments.⁸⁾ The 5'-terminal nucleotides were analyzed as described earlier.³⁾

III. RESULTS AND DISCUSSION

For the primary screening of enzymes, about 1 g of frozen cells were suspended in 10 ml of tris-HCl (pH 7.5) 81 mM 2-mercaptoethanol, disrupted by a sonicator, and centrifuged for 60 min at 30,000 rpm. The cleared supernatant was directly applied onto a small phospho-cellulose column (0.5 cm $\times$ 1 cm), equilibrated with potassium phosphate buffer (pH 7.5)-1 mM 2-mercaptoethanol-5% glycerol (buffer A). After washing the column with 5 ml of buffer A, adsorbed proteins were eluted with 2 ml of 1 M KCl in buffer A. The flow-through fraction and 1 M KCl-eluate were submitted for assay of type II-enzyme activities. All the strains listed in *Table I* were examined by this way, and enzyme activities were detected in fifteen strains.

As the next step, the extracts from the respective strains, in which some enzyme activities were detected, were fractionated by phospho-cellulose column chromatography and enzyme activities in fractions were assayed. The activities in some strains were resolved into two or three different enzymes. The active fractions which generate unique restriction patterns were pooled, and purified by DEAE-cellulose and heparin-agarose columns chromatography. The respective enzymes were concentrated by dialysis against buffer A containing 50% glycerol, and named according to the nomenclature by Smith and Nathans.⁹⁾

Viral and plasmid DNAs (pBR322, pAO2, pAO43, ϕ X174 RF-I, SV40, lambda, and T4dC DNAs) were digested with respective enzymes and the resulting restriction patterns were compared with those of known enzymes. The 5'-terminal sequences of restriction fragments were also analysed. The results of analysis are summarized in *Table I*. Among the eighteen enzymes isolated, *AatII* from *Acetobacter aceti* IFO 12536, *BanII* from *Bacillus aneurinolyticus* IAM1077, *FokI* from *Flavobacterium okeanoikoites* IFO12536 and *MluI* from *Micrococcus luteus* IFO12992 were found to have new specificities. The detailed data for the specificities of these enzymes have been published elsewhere.^{4,10)} The remaining fourteen enzymes were isoschizomers of known enzymes.

We could identify type II enzyme activities only in fifteen strains out of one hundred forty-seven strains examined, but this does not mean that other strains are lacking in this type of enzymes. In our screening procedure, we first fractionated extracts into the adsorbed and non-adsorbed fractions by phospho-cellulose. In

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Table I. Bacterial strains assayed for the presence of type II restriction endonucleases

Bacteria	Sources	Enzymes	Specificity
<i>Acetobacter aceti</i>	IFO 3281	<i>Aat</i> I <i>Aat</i> II	AGG↓CCT isoschizomer of <i>Stu</i> I (11) GACG↓T new enzyme (10)
<i>Acetobacter ascendens</i>	IFO 3188	—	
<i>Acetobacter kuetzingianus</i>	IFO 3222	—	
<i>Acetobacter melanogenes</i>	IFO 3293	—	
<i>Acetobacter tubidans</i>	IFO 3225	—	
<i>Achromobacter georgioplitanum</i>	ATCC 23203	—	
<i>Achromobacter obae</i>	ICR 0870	—	
<i>Achromobacter polymorph</i>	ICR 0880	—	
<i>Achromobacter superficialis</i>	ICR 0890	—	
<i>Acinetobacter species</i>	TOYOBO	—	
<i>Aerobacter aerogenes</i>	AKU 028	—	
<i>Aerobacter aerogenes</i>	IFO 3220	—	
<i>Agrobacterium randiobacter</i>	IAM 1526	—	
<i>Agrobacterium tumefaciens</i>	IAM B-26-1	<i>Atu</i> IAM I	?
<i>Alcaligenes brookeri</i>	IFO 12948	—	
<i>Alcaligenes faecalis</i>	IAM B-141	—	
<i>Alcaligenes faecalis</i>	IFO 3160	—	
<i>Alcaligenes viscolactis</i>	IAM 1517	—	
<i>Arthrobacter atrocyaneus</i>	IFO 12670	—	
<i>Arthrobacter ureafaciens</i>	IAM 625	—	
<i>Bacillus amyloliquefaciens</i> K-50	TOYOBO	—	
<i>Bacillus aneurinolyticus</i>	IAM 1077	<i>Ban</i> I <i>Ban</i> II <i>Ban</i> III	C↓GPyPuCC isoschizomer of <i>Hgi</i> CI (1) GPuGCPy↓C new enzyme (10) AT↓CGAT isoschizomer of <i>Cla</i> I (12)
<i>Bacillus circulans</i>	ICR 1560	—	
<i>Bacillus licheniformis</i>	IFO 12200	—	
<i>Bacillus licheniformis</i>	IAM 11054	—	
<i>Bacillus mesentericus</i> var. <i>flavus</i>	IFO 3028	—	
<i>Bacillus reseus</i> Migula	IFO 3041	—	
<i>Bacterium mycoides</i>	IFO 3040	—	
<i>Brevibacterium ammoniagenes</i>	IFO 12071	—	
<i>Brevibacterium ammoniagenes</i>	IFO 12072	—	
<i>Brevibacterium divaricatum</i>	NRRL 2311	—	
<i>Brevibacterium linens</i>	IFO 12141	—	
<i>Brevibacterium linens</i>	IFO 12147	—	
<i>Brevibacterium protophormiae</i>	IFO 12128	<i>Bpr</i> I	?
<i>Cellulomonas flavigena</i>	IFO 3747	—	
<i>Cellulomonas flavigena</i>	IFO 3754	—	
<i>Cellulomonas flavigena</i>	IFO 12680	—	
<i>Corynebacterium michiganse</i>	ICR 2211	—	
<i>Corynebacterium pseudodiphtheriticus</i>	ICR 2210	—	

<i>Corynebacterium sepedonicus</i>	ICR	2200	—		
<i>Corynebacterium xerosis</i>	IFO	12684	—		
<i>Erwinia aroideae</i>	IFO	3830	—		
<i>Erwinia carotovora</i>	IFO	3057	—		
<i>Escherichia coli crookes</i>	ICR	0010	—		
<i>Escherichia coli</i> E8	ICR	0030	—		
<i>Escherichia coli</i> E9	ICR	0040	—		
<i>Escherichia coli</i> 2bT	ICR	0020	<i>Eco</i> ICRI	GAGCTC	isoschizomer of <i>Sst</i> I (13)
<i>Escherichia coli freudii</i> S-96	ICR	0070	—		
<i>Flavobacterium arborescens</i>	IAM	1100	—		
<i>Flavobacterium lutescens</i>	IFO	3084	—		
<i>Flavobacterium lutescens</i>	IFO	3085	—		
<i>Flavobacterium marginata</i>	IFO	3066	—		
<i>Flavobacterium okeanoikoites</i>	IFO	12536	<i>Fok</i> I	GGATG	new enzyme (4)
<i>Flavobacterium suavealens</i>	IFO	3752	—		
<i>Gluconobacter gluconicus</i>	IFO	3285	<i>Ggl</i> I	?	
<i>Gluconobacter induricus</i>	IFO	3260	<i>Gin</i> I	GGATCC	isoschizomer of <i>Bam</i> HI (14, 15)
<i>Gluconobacter liquefaciens</i>	IFO	12388	—		
<i>Gluconobacter suboxydans</i>	IFO	3130	—		
<i>Gluconobacter suboxydans</i>	IFO	3172	—		
<i>Gluconobacter suboxydans</i> var. α	IFO	3254	—		
<i>Lactobacillus acidophilus</i>	IFO	3205	—		
<i>Lactobacillus arabinosus</i>	IFO	3070	—		
<i>Lactobacillus bulgaricus</i>	IFO	3533	—		
<i>Lactobacillus delbrueckii</i>	IFO	3202	—		
<i>Lactobacillus delbrueckii</i>	IFO	3534	—		
<i>Lactobacillus fermentum</i>	IFO	3071	—		
<i>Micrococcus aurantiacus</i>	IFO	12422	<i>Mau</i> I	CTGCAG	isoschizomer of <i>Pst</i> I (16)
<i>Micrococcus flavus</i>	IFO	3242	—		
<i>Micrococcus glutamicus</i>	ATCC	13032	—		
<i>Micrococcus luteus</i>	IFO	3064	—		
<i>Micrococcus luteus</i>	IFO	12992	<i>Mlu</i> I	A↓CGCGT	new enzymes (4)
<i>Micrococcus luteus</i>	ICR	1850	—		
<i>Micrococcus lysodeikticus</i>	IFO	3333	—		
<i>Micrococcus roseus</i>	IFO	3764	—		
<i>Micrococcus species</i>	AKU	510	—		
<i>Micrococcus species</i>	AKU	520	—		
<i>Micrococcus varians</i>	IFO	3765	—		
<i>Mycobacterium avicem</i>	IFO	3082	—		
<i>Mycobacterium smegatis</i>	IFO	3083	—		
<i>Nocardia erythropolis</i>	IFO	12320	—		
<i>Nocardia erythropolis</i>	IFO	12682	—		
<i>Nocardia mexicana</i>	IFO	3973	—		
<i>Pediococcus homari</i>	IFO	12218	—		
<i>Proteus retgeri</i>	ICR	0650	—		

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<i>Pseudomonas aeruginosa</i>	IFO	3080	—		
<i>Pseudomonas aeruginosa</i>	IFO	3445	—		
<i>Pseudomonas aeruginosa</i>	IFO	3919	—		
<i>Pseudomonas aeruginosa</i>	ICR	3250	—		
<i>Pseudomonas aeruginosa</i>	TOYOBO		—		
<i>Pseudomonas alkaligenes</i>	ATCC	12815	<i>Pai</i> I	GGCC	isoschizomer of <i>Hae</i> III (17)
<i>Pseudomonas alkanolytica</i>	IFO	12319	<i>Pan</i> I	C↓TCGAG	isoschizomer of <i>Xho</i> I (18)
<i>Pseudomonas convexa</i> (Chester)	IFO	3773	—		
<i>Pseudomonas cruciviae</i>	ATCC	3115	—		
<i>Pseudomonas decanhae</i>	IAM	1048	—		
<i>Pseudomonas destrolytica</i>	IFO	12570	—		
<i>Pseudomonas diminuta</i> (Leifson)	IFO	12697	—		
<i>Pseudomonas fluorescens</i>	IFO	3081	—		
<i>Pseudomonas fluorescens</i>	IFO	3507	<i>Pfl</i> I	?	
<i>Pseudomonas fluorescens</i>	IAM	1057	—		
<i>Pseudomonas groveolons</i> (<i>taetrolens</i>)	IFO	3460	—		
<i>Pseudomonas iodium</i>	IFO	3558	—		
<i>Pseudomonas maltophilia</i>	IFO	12690	—		
<i>Pseudomonas marginalis</i>	IFO	3925	—		
<i>Pseudomonas ovalis</i>	IFO	3738	—		
<i>Pseudomonas ovalis</i>					
<i>Pseudomonas polycolor</i>	IFO	3918	—		
<i>Pseudomonas putida</i>	ATCC	12533	—		
<i>Pseudomonas putida</i> C-83	TOYOBO		<i>Ppu</i> I	GGCC	isoschizomer of <i>Hae</i> III (17)
<i>Pseudomonas putida</i> C-175	TOYOBO		—		
<i>Pseudomonas riboflavina</i> (Foster)	IFO	3140	—		
<i>Pseudomonas salanacerium</i>	IFO	3509	—		
<i>Pseudomonas striata</i>	IFO	12996	—		
<i>Pseudomonas stutzeri</i>	IFO	12695	—		
<i>Pseudomonas syncyanae</i>	IFO	3757	—		
<i>Pseudomonas synxantha</i>	IFO	3757	—		
<i>Pseudomonas species</i> AK-LPL-7	TOYOBO		—		
<i>Pseudomonas tobaci</i>	IFO	3508	—		
<i>Sarcina aurantiaca</i>	IFO	3046	—		
<i>Sarcina lutea</i>	IFO	3232	—		
<i>Sarcina lutea</i>	IFO	1099	—		
<i>Serratia marcescens</i>	IFO	3046	—		
<i>Serratia marcescens</i>	IFO	3054	—		
<i>Serratia plymuthium</i>	IFO	3055	—		
<i>Staphyrococcus aureus</i>	IFO	3082	—		
<i>Staphyrococcus faecalis</i>	IFO	3181	—		
<i>Staphyrococcus faecalis</i>	IFO	3826	—		
<i>Staphyrococcus lactis</i>	IFO	2546	—		
<i>Streptomyces adonifer</i>	ATCC	6246	—		
<i>Streptomyces albus</i>	IFO	3418	—		
<i>Streptomyces albus</i>	IFO	3422	—		
<i>Streptomyces albus</i>	IFO	3710	—		

<i>Streptomyces albus</i>	IFO	13011	—		
<i>Streptomyces caudatus</i>	ATCC	13077	—		
<i>Streptomyces cellulosae</i>	IFO	3713	—		
<i>Streptomyces flaveolens</i>	IAM	0005	—		
<i>Streptomyces griseus</i>	IAM	0084	—		
<i>Streptomyces intermedius</i>	ATCC	3329	—		
<i>Streptomyces longisporus</i>	ATCC	14697	—		
<i>Streptomyces microflavus</i>	IFO	13062	—		
<i>Streptomyces parourascens</i>	IFO	13077	—		
<i>Streptomyces phaeochromogenes</i>	IFO	3108	<i>Spa</i> I	GCATGC	isoschizomer of <i>Sph</i> I (19)
<i>Streptomyces purpeofusoeus</i>	IFO	12905	—		
<i>Streptomyces resistomyceficus</i>	ATCC	19804	—		
<i>Streptomyces resistamyceficus</i>	ATCC	19807	—		
<i>Streptomyces rochei</i>	IFO	12908	—		
<i>Streptoverticillium netropsis</i>	IFO	3723	—		
<i>Streptoverticillium netropsis</i>	IFO	12893	—		

Footnotes to Table:

1. Abbreviations for the sources of strains. AKU: Faculty of Agriculture, Kyoto University, ATCC: American Type Culture Collection, Rockville, Maryland, USA., IAM: Institute for Applied Microbiology, University of Tokyo, ICR: Institute for Chemical Research, Kyoto University, IFO: Institute for Fermentation, Osaka, NRRL: Northern Utilization Research and Development Division, US Department of Agriculture, Peoria, Illinois, USA, TOYOBO: Toyoboseki Co.
2. Recognition sequences are written from 5' to 3', only one strand being given, and when known, the site of cleavage is indicated by the arrow.
3. Mode of cleavage of *Fok* I is as follows: (5') ... GGATG NNNNNNNNNN↓
(3') ... CCTAC NNNNNNNNNNNN↑

this fractionation, cellular DNA and RNA and most of proteins including non-specific nucleases are recovered in the flow-through (non-adsorbed) fraction, and this would make it difficult to detect activities of specific enzymes in the fractions. It is quite likely that we are missing enzymes with poor affinity toward phospho-cellulose. Nevertheless, we could isolate eighteen species of enzymes in which four were found to be new enzymes. A common cloning vector pBR322 contains a single cutting site of *Aat*I in the upstream region of the *bla* gene, and two cutting sites of *Ban*II, but separated by fourteen base-pairs, in the *tet* gene. *Mlu*I does not cleave this plasmid. *Fok*I was a new type of enzyme which generates a protruding 5'-end of four nucleotides at the site nine nucleotides downstream from the recognition sequence. These enzymes would become powerful tools for gene engineering. The remaining fourteen enzymes were isoschizomers of known enzymes, but some of enzymes (*e.g.* *Eco*ICRI, *Ban*I, *Spa*I, *Gin*I, *Aat*I, *Mau*I and *Ggl*I) were very high in yields and could be easy to purify. Some of the originally reported enzymes may be replaced by these enzymes.

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